Neuroprotective effect of hesperidin on pyrethroid pesticide induced oxidative stress response and β-amyloid toxicity in *Caenorhabditis elegans*

A

SYNOPSIS

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Submitted by

Krishna Rana

Under the supervision of

Dr. Priyanka Gautam

Assistant Professor
Department of Zoology
Faculty of Science

DEPARTMENT OF ZOOLOGY, FACULTY OF SCIENCE
DAYALBAGH EDUCATIONAL INSTITUTE (DEEMED UNIVERSITY)
DAYALBAGH, AGRA, 282005

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1.0. INTRODUCTION

Impaired functioning of neuronal signaling pathway due to improper collapse and accretion of some specific protein causes neuron degeneration which causes the progressive loss of the structure and function of neurons that eventually leads to neuronal death. Alzheimer's, Parkinson's, Huntington's and Amyotrophic Lateral Sclerosis are the most leading neurodegenerative diseases which ensue as a consequence of neurodegeneration processes (Hesp, *et al*., 2015).

Alzheimer's disease (AD) is the most common cause of dementia in the aging, accounting for 60–70% cases of progressive cognitive impairment. Histopathological changes in Alzheimer's disease include neuronal cell death, formation of amyloid plaques (aggregates of beta amyloid) and neurofibrillary tangles (hyperphosphorylated tau protein) (Hureau and Faller, 2009). The genetic studies suggest that the death of cells in Alzheimer's is due to oxidative stress, mitochondrial dysfunction and protein aggregation. Aberrant aggregation of beta amyloid ($A\beta_{1-42}$) is a key characteristic of Alzheimer's disease, (Drake, *et al*., 2003) which can be caused by different ways like mutations in related genes and through various environmental toxicants such as pesticides, herbicides, fungicides. Now these days, the excessive usage of Pesticides such as organophosphates, pyrethroids and carbamates are the part of house and agriculture, which leads to potential threat to human health and other living beings (Yadav, *et al*., 2016). Pesticides have a broad range of insecticides out of these we have selected pyrethroid pesticide. These are the pesticide used most commonly in various commercially available products to control pest. These are the neurotoxin that attacks the nervous systems of insects as well as humans (Melstrom, 2007; Zhu, *et al*., 2020).

Recently, there has been heightened interest in assessing the role of oxidative stress in neurologic disorders. Oxidative stress speaks to an irregularity between toxic reactive oxygen species (ROS) and antioxidant systems. There are evidences that free radicals (reactive oxygen species (ROS)) play an important role in pathogenesis of Alzheimer's. Significant levels of ROS causes inflammation and cytotoxicity, harming proteins, lipids, DNA, disturbing cellular functions and causing neuronal cell death (Meng, *et al.,* 2017). The oxidative damage theory proposes that accumulation of molecular damage because of reactive oxygen species (ROS), particularly superoxide ($O_2^{\bullet-}$) and its derivatives, is a primary cause of oxidative stress. Superoxide ($O_2^{\bullet-}$) changed into hydrogen peroxide (H_2O_2) by

superoxide dismutase (SOD). Hydrogen peroxide (H_2O_2) subsequently converted into H_2O and O_2 by various antioxidant enzymes like catalase (CAT), glutathione peroxidase (GPx), superoxide dismutase (SOD) and peroxiredoxins (Prxs) (Thapa and Caroll, 2017; Ighodaro and Akinloye, 2018) among these, superoxide dismutase (SOD) is a major antioxidant enzyme that protects against oxidative stress by catalyzing the removal of superoxide ($O_2^{\bullet-}$) (Honda and Honda, 1999).

Various studies have been conducted to study the neurodegenerative disease on soil nematode, *Caenorhabditis elegans*. Several characteristic features of this free living worm making it a suitable model organism includes:- Small transparent body (~1 mm in length) with Short life cycle (~3-4 days) and life span (~3 weeks) with high reproducible rate (Brenner, 1974). Adult hermaphrodite contains 302 neurons (Dimitriadi and Hart, 2010). Completely sequenced genome with 65% orthologous genes of human-related diseases (Shen, *et al*., 2018) with similar neurotransmitters and receptors (Meyer and Williams, 2014) Make them an immense model to study neuroscience, oxidative stress response, cell death, aging, toxicology, and developmental biology.

In *C. elegans*, the ROS protection mechanism is controlled by DAF-16 a FoxO (Forkhead box protein O) transcription factor (orthologous of mammals (human) FOXO3 gene) through c-Jun N-terminal Kinase (JNK) Pathway and the insulin/IGF-1 signaling (IIS) pathway (Wolf, *et al*., 2007). JNK and IIS pathways are well conserved across mammals and nematodes for investigating the process of aging and oxidative stress resistance regulation (Zhao, *et al*., 2017). In activated IIS pathway, chained phosphorylation of DAF-16 keeps it in cytoplasm in the inactive form (Wolf, *et al.,* 2007). In contrast, under stressed conditions and mutational changes, this pathway promotes the de-phosphorylation of DAF-16, allowing its entry into the nucleus due to which they act as transcription factors (Rodriguez, *et al.,* 2012; Murphy and Hu, 2013). Further DAF-16 up-regulates the gene expression of superoxide dismutase-3 (SOD-3), that in return triggers the activity of mitochondrial antioxidant system to prevent oxidative stress by catalyzing the removal of Superoxide ($O_2^{\bullet-}$) that may be inhibited amyloid-beta aggregation (Du, *et al.,* 2019).

Antioxidant approaches for the prevention and therapy of Alzheimer's disease are of central interest. Experimental as well as clinical data shows that natural antioxidants like vitamin E, estrogens, and plant flavonoids have occurred anti-oxidative properties that prevent oxidative stress by reducing the

production of ROS and can be inhibited amyloid-beta aggregation also (Behl, 1999; Hajialyani, *et al*., 2019). Antioxidants have an important role in preventing a variety of lifestyle-related diseases and aging because these are closely related to the active oxygen and lipid peroxidation. Hesperidin is a naturally occurring flavonoid that represents one of the most ubiquitous classes of phenolic compounds that exists in citrus fruits and other plants (Kim, *et al*., 1998). It can be isolated in large amounts from the peels of *Citrus aurantium* (bitter orange), *Citrus sinensis* (sweet orange), and *Citrus unshiu* (Satsuma mandarian) (Tamilselvam, *et al*., 2013). It demonstrated excellent antioxidant activity by blocking and/or scavenging free radicals. Many scientific studies and experiments have manifested that hesperidin has anti-stress, anti-oxidative, antipyretic, analgesic, immune-modulatory, anti-inflammatory, anti-diabetic properties that have been proven against neuropathic pain, ulcer protective and various cancerous studies (Pandey, *et al*., 2018; Hajialyani, *et al*., 2019). So, in the present study, we have selected hesperidin as a natural flavonoid.

The study will be focused on testing of neuroprotective effect of hesperidin to evaluate the downregulation of oxidative stress as well as further aggregation of beta-amyloid. The excessive use of various pesticides in commercially products to which humans may expose directly or indirectly and causing oxidative stress that could be a leading cause of neurodegenerative disease, Alzheimer's. The study will be conducted using *C. elegans*, a nematode as a model organism. The study will help in improving public health by achieving a healthy life with a healthy mind.

2.0. REVIEW OF LITERATURE

Literature available on the Alzheimer's disease, pesticides toxicity and prevention studies have been reviewed and presented. Various experiments were carried out by scientists to study the phyto-protective effects of several forms of drugs and natural products on *C. elegans*.

Alois Alzheimer (1907) first described "a peculiar disease" in a case of Auguste D. a patient who had profound memory loss, unfounded suspicions about her family, and other worsening psychological changes. **Emil Kraepelin (1910)** a German psychiatrist who worked with Dr. Alois Alzheimer, first named the disease as "Alzheimer's Disease" in the eighth edition of his book "Psychiatrie" (http://www.alz.org/research/science/major_milestones_in_alzheimers.asp). In 1974, **Brenner** observed that the nematode *C. elegans* has small body size of ~1 mm is the length with a short life

cycle (3 days) and describe that how the *C. elegans* can be grown in *in-vivo* condition using NGM media and *E.coli* OP50 as food source. **Glenner and Wong (1984)** reported identification of "a novel cerebrovascular amyloid protein," known as beta-amyloid — the chief component of Alzheimer's brain plaques and a prime suspect in triggering nerve cell damage. **Williams and Dusenbery (1988)** determined that acute lethality of metals using the nematode *C. elegans* and the LC50 values were compared to predict mammalian LC50 values for same matels, again **Williams and Dusenbery (1990)** determined that *C. elegans* is usefulness as an aquatic test species. **Markesbery (1997)** reviewed that oxidative stress hypothesis in Alzheimer's disease, concluded that free radicals are possibly involved in the pathogenesis of neuron death in AD. *C. elegans* was the first multicultural organism that has completely sequenced genome reported by **the *C. elegans* Sequencing Consortium (1998)**. **Link, *et al.*, (1999)** showed that direct observation of stress response in *Caenorhabditis elegans* using a reporter transgene. **Varadarajan, *et al*., (2000)** reviewed that oxidative stress manifested by protein oxidation and lipid peroxidation, among other alterations is a characteristic of AD brain and involvement of free radicals and ROS are an essential factor in the Pathogenesis of AD **(Christen, 2000)** and cause of nerve cell dysfunction and death **(Vickers, *et al.*, 2000)**. **Link, *et al.*, (2003)** innovated the transgenic *Caenorhabditis elegans* Alzheimer's disease model to expression analysis of gene orthologous to the human beta amyloid peptide (Aβ), while **Kitagawa, *et al.*, (2003)** examined the role of the PS1 homologue gene sel-12 of *Caenorhabditis elegans* under oxidative stress and clarified the sel-12-induced apoptosis.

Further **Cole, *et al.*, (2004)** explored the nematode *Caenorhabditis elegans* as model of organophosphate-induced mammalian neurotoxicity tested by computer tracking for their acute behavioral toxicity, whereas **Oh, *et al.*, (2005)** demonstrated that jnk regulates lifespan in *Caenorhabditis elegans* by modulating nuclear translocation of forkhead transcription factor/daf-16. Therapeutic potential for prevention of Aβ associated neurodegenerative disorders by a specific soy isoflavone glycitein suppress Aβ toxicity through combined anti-oxidative activity and inhibition of Aβ deposition determined by **Gutierrez-Zepeda, *et al.*, (2005)**. **Nunomura, *et al.*, (2006)** suggested that oxidative stress is mechanistically and chronologically associated with other key features of AD, namely, metabolic, mitochondrial, metal, and cell-cycle abnormalities. **Schmidt, *et al.*, (2007)**

describe that *C. elegans* can be used for understanding of the genetic mechanisms associated in neurodegenerative disorders. While **Rajini, *et al.*, (2008)** evaluated the *Caenorhabditis elegans* using lethality, movement, and acetylcholinesterase (AChE) activity as the endpoints after exposure of organophosphorus (OP) insecticides. **Hureau and Faller (2009)** reviewed how the Aβ peptide is oxidized during ROS production and different pathways for the ROS generation by the Cu (I/II)-Aβ species, and human tau in *C. elegans* becomes highly phosphorylated and exhibits conformational changes similar to PHP tau and human PHF tau, both, wt tau and PHP tau induced a progressive age-dependent development of a phenotype of uncoordinated locomotion (unc) in the absence of neuronal degeneration assessed by **Brandt, *et al.*, (2009)**.

Consequently, antioxidant approaches for the prevention and therapy of AD are of crucial curiosity. Many studies suggested that natural antioxidants, such as vitamin E, flavonoids and estrogens, are neuroprotective and may help patients suffering from AD. **Uttara, *et al.*, (2009)** revealed that recognition of upstream and downstream antioxidant therapy to oxidative stress has been proved an effective tool in alteration of any neuronal damage as well as free radical scavenging, oxidative stress and amyloid-β peptide. The effects of EGCG on lifespan and stress resistance in wild-type N2 and transgenic strains of *Caenorhabditis elegans* investigated by **Abbas and Wink (2009)** again, **Abbas and Wink (2010)** demonstrated that green tea and EGCG protects against ROS-mediated and age-related diseases. **Liao, *et al.*, (2011)** analyzed that lifespan extension by curcumin *C. elegans* is attributed to its anti-oxidative properties but not its antimicrobial properties. Flavonoids structure–activity relationship in vivo used a comparative approach by exposing *C. elegans* to the structurally related flavonoids myricetin, quercetin, kaempferol and naringenin, and their impact on lifespan and on putative modes of action assessed by **Grunz, *et al.*, (2012)**.

The neuro-protective effects of hesperidin, a citrus fruit flavanol, against rotenone-induced apoptosis in human neuroblastoma SK-N-SH cells investigated by **Tamilselvam, *et al.*, (2013)**. **Bigoniya and Singh (2014)** explored that hesperidin prevents oxidative cell injury by significant rise of super oxide dismutase, glutathione and catalase levels in gastric mucosa in wistar rat. **Asthana, *et al.*, (2015)** evaluated that the protective effect of *Ocimum basilicum* and *Premna integrifolia* on lifespan and stress response in *Caenorhabditis elegans*. The transcription factor DAF-16/FOXO was involved in both

oleic acid- and *C. tenuifolia* seed oil-mediated oxidative stress resistance in *C. elegans* confirmed by **Wei, *et al.*, (2016). Habibyar, *et al*., (2016)** suggested that hesperidin have strong memory enhancing and antioxidant effects, it can be considered as a potential candidate for its further pharmacological evaluation for AD-induced dementia. GNC extends the lifespan and stress resistance properties and explored the associated regulatory mechanism and enhances its resistance to oxidative stress via a daf-16/FOXO- dependent pathway, using a *Caenorhabditis elegans* model investigated by **Meng, *et al.*, (2017). Arantes, *et al.*, (2018)** contributed that comprehension of guarana effects in vivo, useful to inhibit or treat aging associated disorders, and also suggest purinergic signaling as a possible therapeutic target for longevity studies. **Pandey, *et al.*, (2018)** suggested that hesperidin can be considered as a potential anti-cancerous compound for the treatment of gall bladder carcinoma in human. **Du, *et al.*, (2019)** suggested that MCCP has a significant protective effect on β-amyloid-induced toxicity in *C. elegans* by reducing β-amyloid aggregation and inducing DAF-16 nuclear translocation that activate the downstream signal pathway and enhance resistance to oxidative stress. Protective effects of hesperidin on liver, kidney, heart, and age-related memory impairment, antimicrobial, anticancer, antihypertensive and antiulcer explained by **(Ganeshpurkar and Saluja (2019)**. **Hajialyani, *et al*., (2019)** suggested that Hesperidin-enriched dietary supplements can significantly improve cerebral blood flow, cognition, memory performance and neuro-protective effect against neurodegenerative diseases. **Wang, *et al.*, 2020** evaluated that potential effects of orange extracts on longevity, health span, and improved stress resistance against thermal and UV-B stresses, motility, and antioxidant-related enzyme activities (SOD and CAT) in *Caenorhabditis elegans* model**.**

3.0. OBJECTIVES

- Screening of pyrethroid pesticide for inducing oxidative stress in *C. elegans.*
- Protective effect of hesperidin on physiological changes in *C. elegans*.
- Qualitative and Quantitative analysis of the protective effect of hesperidin in *C. elegans*.
- Gene expression analysis of oxidative stress regulatory gene in *C. elegans*.

4.0. MATERIALS AND METHODS

4.1. MATERIALS

A wild type N2 Bristol along with transgenic strains of *C. elegans* CL4176, CF1553 and TJ356 will be used for the present study. CL2120 expresses human Aβ $_{1-42}$ in muscle cells, CF1553 will be used to examine the expression of SOD-3 whereas, TJ356 will be used to test DAF-16 localization. All strains will be procured from the Caenorhabditis Genetics Center (CGC) (University of Minneapolis, MN, USA). The worms will be culture on NGM plate pre-seeded with the bacterial culture of *E. coli* OP50 strain, which will also be procured from CGC.

The pyrethroid pesticide will be used for toxicity assessment. These are the most common pesticide used in commercially available products, will be purchased from Sigma-Aldrich (St. Louis, USA). The hesperidin a natural flavonoid will be used to visualize neuroprotective effect, will be procured from TCI (Tokyo Chemical Industry) chemicals (India) Pvt. Ltd. All other chemicals will be used of analytical grade.

4.2. METHODS

4.2.1. Culture of Worms

All strains will be cultured following the methodology of Brenner, 1974, at 20°C on NGM (nematode growth media) pre-seeded with *E. coli* OP50 as a food source (Leung *et al*., 2008). Synchronization of nematode cultures will be achieved by hypochlorite treatment of gravid hermaphrodites and eggs will be allowed to hatch overnight on NGM plate (Arantes *et al*., 2018).

4.2.2. Pesticide Dose Determination by LC50

Age synchronized L4 stage worm will be transferred to NGM plates with desired concentration of pesticide. To determine the dose of pesticide. Pesticidal toxicity will be achieved by LC50, (concentration at which there is a 50% reduction in number of worms compared to controls). It will be calculated by the method of probit analysis with a log transformation (Williams and Dusenbery 1990, Kucukgul *et al*., 2013).

4.2.3. Free Radicals Scavenging Activity of Hesperidin

The antioxidant activity of hesperidin will be measured in term of hydrogen donating or free radical scavenging ability using the stable DPPH radical (1,1-diphenil-2-picrylhydrazyl). Experiments will be carried out according to method described by Blois, 1958 (Thabit, *et al.*, 2018). The percentage inhibition of the DPPH radical will be calculated using the following formula:

$$I\ \% = \frac{Ao - A}{Ao} \times 100$$

Where, I = DPPH inhibition (%), A0 = absorbance of control sample (t = 0 h) and A = absorbance of a tested sample at the end of the reaction (t = 1 h).

4.3. PHYSIOLOGICAL PARAMETERS

Oxidative Stress is considered as a major risk factor for highly prevalent age-related neurodegenerative diseases. AD is an insidious progressive age-related neurological disorder that leads to the loss of cognitive functions, memory processes, and associated physiological changes (Yadav *et al*., 2016). So we will analyze physiological changes followed by various qualitative, quantitative parameters as well as molecular analysis to justify protective effect of hesperidin on oxidative stress leading to Alzheimer's.

All parameters used in the study will be analyzed for the pesticide toxicity as well as to see the protective effect of hesperidin.

4.3.1. Paralysis

Paralysis will be determined by counting the number of stable worms, usually registering a response when touched by platinum picker (Hassan *et al*. 2009).

4.3.2. Learning

The learning behavior in *C. elegans* will be studied by keeping the treated nematodes in the center of the NGM plate, having holes at equidistance from the center filled with bacterial food suspension (*E. coli* OP50) and toxic solution. (Tejeda-Benitez, and Olivero-Verbel, 2016).

4.3.3. Lifespan

The worms will be cultured and propagated in NGM pre-seeded with *E. coli* OP50 at 20 °C. Age-synchronized worms will be transferred onto new NGM plates with desired concentration of pesticide. Treated worms will be transferred every day onto new NGM plates with desired concentration of hesperidin. Surviving and dead worms will be counted daily until all dead. The survival rate will be calculated by dividing the number of live nematodes by the total number of nematodes (Tejeda-Benitez, and Olivero-Verbel, 2016; Xu *et al*., 2019).

4.4. QUALITATIVE PARAMETERS

Qualitatively, the post exposure changes in pesticide and hesperidin treated worms will be analyzed with help of GFP tagged reporters gene such as SOD-3, DAF-16 and unc-17, eat-4 for oxidative stress and neuron-degeneration respectively.

4.4.1. Superoxide Dismutase (SOD)-3 Expression

The CF1553 Strains of SOD-3::GFP fosmid reporter will be used for SOD-3 expression. Age synchronized L4 stage worms will be transferred on NGM plates with desired concentration. Exposed worms will be mounted onto the slide with sodium azide. Up-regulation and down-regulation of SOD-3 expression will be analyzed with a fluorescence microscope equipped with a digital camera. Images will be analyzed by image analysis software (Du *et al.,* 2019).

4.4.2. DAF-16 Localization

The TJ356 Strains of DAF-16::GFP fosmid reporter will be used to examine the intracellular distribution of DAF-16. Age synchronized L4 stage worms will be transferred on NGM plates with desired concentration. Exposed worms will be mounted onto the slide with sodium azide. The up-regulation of the expression of DAF-16::GFP for nuclear localization from cytoplasm due to oxidative stress will be analyzed with a fluorescence microscope equipped with a digital camera. Images will be analyzed by image analysis software (Oh *et al*., 2005; Abbas and Wink 2010).

4.4.3. Neurodegeneration

Alzheimer's disease is a progressive neurodegenerative disorder associated with the loss of cholinergic and glutamatergic neurons and the formation of excessive plaques containing amyloid β protein aggregation. Transgenic strain OH13083 of unc-17::GFP fosmid reporter and OH11124 for eat-4::GFP fosmid reporter will be used to visualize the degeneration of the cholinergic and glutamatergic neurons respectively (Kratsios *et al*., 2012). The exposed worms will be analyzed using dissecting fluorescence microscope by immobilizing with 2% of sodium azide, pre-dropped on 2% agarose (Nass *et al*., 2002). The results of neuron degeneration will be further analyzed by image analysis software (Estevez *et al*., 2012).

4.5. QUANTITATIVE PARAMETERS

Oxidative stress (OS) leading to free radical attack on neural cells contributes destructive role to neuron-degeneration. Quantitative studies will be done for evaluating the ROS and Aβ aggregation to analyze oxidative stress.

4.5.1. ROS Determination

The effect of hesperidin on ROS levels in *C. elegans* under oxidative stress conditions induced by pesticide will be measured using 2'7'-dichlorodihydrofluoroscein diacetate (H_2DCFDA). It is a cell-permeable dye, which is converted to highly fluorescence compound 2'7' dichlorodihydrofluoroscein (DCF) due to the presence of reactive oxygen species (ROS). Pesticide and hesperidin treated worms will be transferred to M9 buffer containing H_2DCFDA for 3 h at 20 °C (Meng *et al*., 2017). After incubation, the worm will be washed two times with m9 buffer to remove unbounded H_2DCFDA, then the worms will be transferred to 96-well on microplate reader for ROS quantification (Du *et al*., 2019).

4.5.2. Aβ Aggregation

Transgenic nematodes CL4176 (expressing Aβ in body wall muscle cells) will be fixed in 4% paraformaldehyde for 24 h at 4^{o}C and then permeabilized in 5% β-mercaptoethanol, 1% triton X-100, 125 mM tris, at 37^{o}C in the incubator for 24 h. After washing 2 times with M9 buffer, stained with 0.125% thioflavin T (ThT) and destained with sequential ethanol washes. The worms will be washed with PBS and will be transferred to 96 well plates (Gutierrez-Zepeda *et al.*, 2005). Aggregation of Aβ *in vivo,* the fluorescence intensity will be subsequently measured using a microplate reader. (Xu *et al*., 2019).

4.6. MOLECULAR PARAMETERS

4.6.1. Gene Expression

DAF-16 is the oxidative stress resistance regulatory gene that regulate the ROS production by SOD enzymatic activity on free radicals. Gene expression changes in oxidative stress regulatory gene (DAF-16) in *C. elegans,* will be analyzed by two step Reverse Transcription Polymerase Chain Reaction (RT-PCR). First step includes the extraction of whole RNA content from the nematode worm. Second step includes cDNA synthesis from RNA. Further the DNA will be amplified by RT-PCR using gene specific primers and the amplified product will be analyzed by Agarose Gel Electrophoresis (Alexander *et al.*, 2014).

5.0. PLAN OF WORK

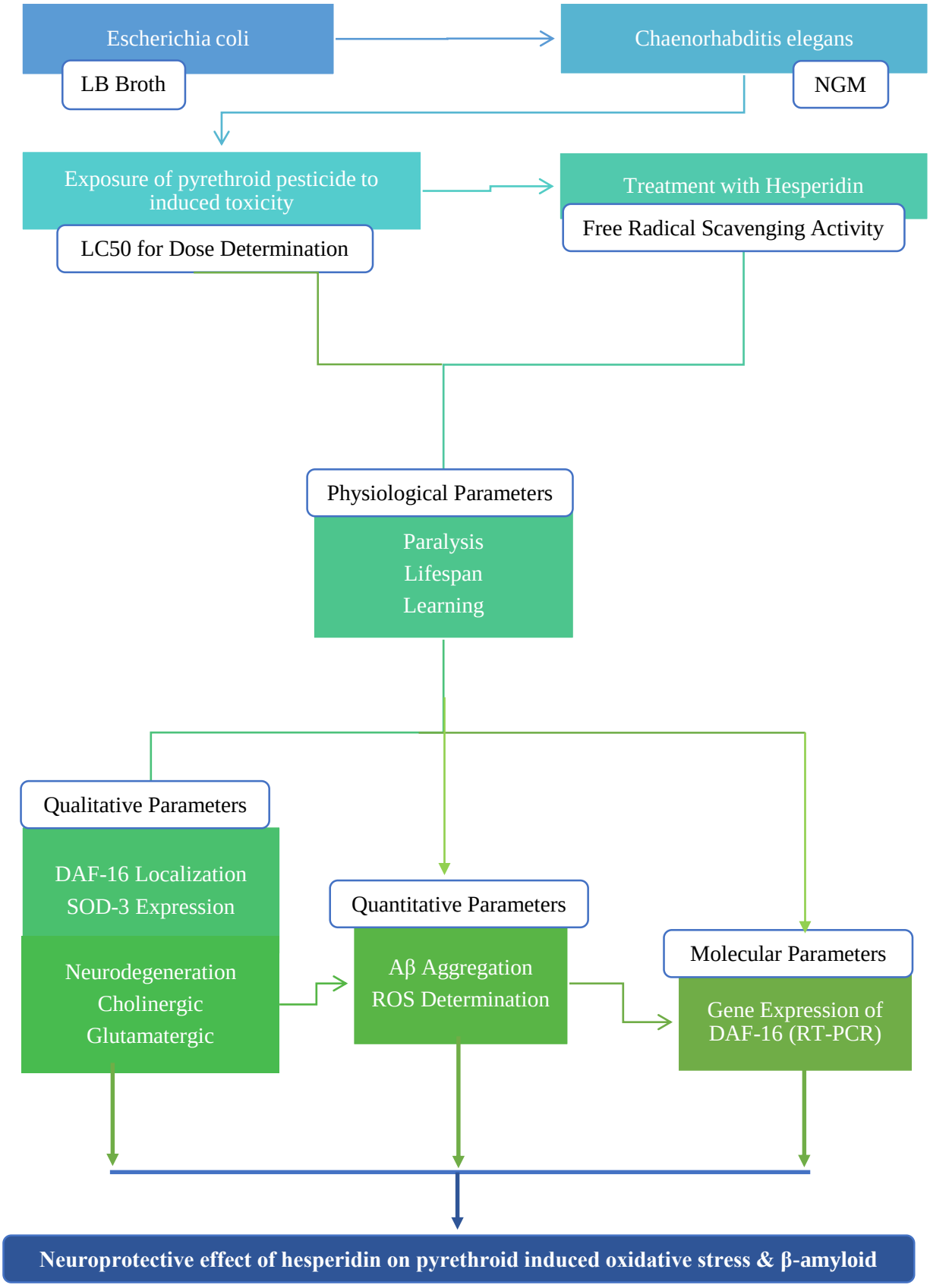

Fig.1. Plan of work.

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